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## Pediocin PA-1, a Bacteriocin from *Pediococcus acidilactici* PAC1.0, Forms Hydrophilic Pores in the Cytoplasmic Membrane of Target Cells

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**Pediocin PA-1 is a bacteriocin which is produced by *Pediococcus acidilactici* PAC1.0. We demonstrate that pediocin PA-1 kills sensitive *Pediococcus* cells and acts on the cytoplasmic membrane. In contrast to its lack of impact on immune cells, pediocin PA-1 dissipates the transmembrane electrical potential and inhibits amino acid transport in sensitive cells. Pediocin interferes with the uptake of amino acids by cytoplasmic membrane vesicles derived from sensitive cells, while it is less effective with membranes derived from immune cells. In liposomes fused with membrane vesicles derived from both sensitive and immune cells, pediocin PA-1 elicits an efflux of small ions and, at higher concentrations, an efflux of molecules having molecular weights of up to 9,400. Our data suggest that pediocin PA-1 functions in a voltage-independent manner but requires a specific protein in the target membrane.**

Some lactic acid bacteria produce antimicrobial substances called bacteriocins (24). Several bacteriocins have been isolated and purified, but in only a few cases has the mode of action of these compounds been determined (26). Nisin (5, 14, 27, 40), lactostreptin 5 (50), and lactococcin A (46) all act on the cytoplasmic membrane of target cells. Although poorly understood, the molecular mechanisms by which these bacteriocins function differ. Many lactic acid bacteria are used in food fermentation, and because of their bacteriocin production, these strains are used as natural food preservatives. Nisin is the only bacteriocin which is commonly used as a purified substance for food preservation (38).

The genus *Pediococcus* comprises a large group of lactic acid bacteria that are used commercially in meat (44) and vegetable (35) fermentations. *Pediococcus* strains that produce bacteriocins are good candidates for food processing and at the same time function in the preservation of processed and fresh food (1, 32, 33). Purified pediocin has been shown to be a good food preservative (49). Recently, it has been suggested that *Pediococcus* strains might also be used as silage inoculants (13).

In some of the bacteriocin-producing *Pediococcus* species the genes determining bacteriocin production have been localized on plasmids (8, 18, 19, 22, 32, 39). In *Pediococcus acidilactici* PAC1.0, a gene cluster which includes the structural pediocin gene and genes for putative determinants of the pediocin secretion and immunity mechanisms was local-

ized on a 9.0-kbp plasmid. These genes have been cloned and sequenced (32). Pediocin PA-1 has been purified to homogeneity, and its primary structure has been determined by Edman degradation (20). Pediocin PA-1 is synthesized as a 62-amino-acid precursor and is cleaved behind two glycine residues at a conserved cleavage (processing) site found in several bacteriocins (30, 32). Mature pediocin PA-1 is a highly hydrophobic, positively charged peptide consisting of 44 amino acids (20). The protein contains two disulfide bonds (20). Pediocin PA-1 inhibits the growth of various bacteria, including *Listeria monocytogenes* (20, 31, 37), which frequently causes food-borne listeriosis (42, 48).

In this paper we describe the mode of action of pediocin PA-1. We found that pediocin PA-1 acts on the cytoplasmic membrane of target cells by forming voltage-independent pores which cause an efflux of important cellular metabolites.

### MATERIALS AND METHODS

**Bacterial strains and media.** *P. acidilactici* PAC1.0 (18) and *Pediococcus pentosaceus* PPE1.2 (17) were grown in MRS broth or on MRS agar (Difco Laboratories, Detroit, Mich.) at 30°C without aeration. For maintenance of the pediocin plasmids, the cells were grown in the presence of 5 µg of erythromycin per ml.

**Determination of pediocin activity and production level.** Pediocin production in a sample was determined as described previously (20); 1 arbitrary unit (AU) of pediocin PA-1 activity was defined as the maximum dilution of a pediocin-containing sample that caused a clearly visible zone of inhibition in a sensitive *P. pentosaceus* PPE1.2 test culture overlay.

**Purification of pediocin PA-1 and pediocin-like bacteriocins.** Pediocin PA-1 was purified from the supernatant of *P. acidilactici* PAC1.0 after overnight growth in CGB broth at

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30°C without aeration (2). The bacteriocin was concentrated from the supernatant by ethanol precipitation and was purified by preparative isoelectric focusing on a Rotofor cell (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the supplier. Sakacin P and sakacin A (curvacin A) were isolated and purified as described elsewhere (30, 45).

**Measurement of  $\Delta\psi$ .** The transmembrane electrical potential ( $\Delta\psi$ ) in intact cells was determined with an electrode specific for the lipophilic cation tetraphenylphosphonium as described previously (43).

**Transport assays.** Amino acid transport by *Pediococcus* cells was analyzed as described previously for *Lactococcus lactis* (46, 47).  $1\text{-}^{14}\text{C}$ -labeled 2- $\alpha$ -aminoisobutyric acid (AIB) (59 mCi/mmol) and L-[U- $^{14}\text{C}$ ]glutamate (285 mCi/mmol) were used at final concentrations of 8.5 and 1.75  $\mu\text{M}$ , respectively. Uptake of L-[U- $^{14}\text{C}$ ]leucine by membrane vesicles was analyzed in the presence of an imposed proton motive force or by measuring counterflow as described previously (46).

**Isolation of lipids and preparation of CF-containing liposomes.** *P. pentosaceus* lipids were extracted (9) and washed with acetone-ether as described previously (23). Unilamellar liposomes with an average diameter of 100 nm were prepared by the method of Goessens et al. (16). Lipids dissolved in chloroform-methanol (9:1, vol/vol) were thoroughly dried under a vacuum for 1 h. Traces of solvent were removed under a stream of  $\text{N}_2$ . The dry lipid was suspended (6 mg/ml) in a buffer containing 50 mM carboxyfluorescein (CF) and 50 mM K-(2-[N-morpholino]ethanesulfonic acid) (K-MES) (pH 6.0). The lipid suspension was dispersed by ultrasonic irradiation with a bath-sonicator (Sonicor; Sonicor Instruments, New York, N.Y.). Liposomes were obtained by five cycles of freezing in liquid nitrogen and thawing in water at room temperature and were subsequently extruded through 0.4-, 0.2-, and 0.1- $\mu\text{m}$ -pore-size polycarbonate filters (Nuclepore Co., Pleasanton, Calif.) (two times each) by using an extrusion device (Lipex Biomembranes, Vancouver, British Columbia, Canada). Nonencapsulated CF was removed from the liposomes by gel filtration on Sephadex G-75 in 50 mM K-MES. The liposomes were stored on ice until they were used.

**Preparation of membrane vesicles and fusion with liposomes.** Membrane vesicles of pediocin-sensitive and immune *P. pentosaceus* cells were prepared by using the procedure described by Driessen (9). The membrane vesicles (1 mg of protein) were mixed with liposomes composed of *P. pentosaceus* lipids (13.6  $\mu\text{mol}$  of phospholipid) in 50 mM K-MES buffer (pH 6.0) and fused by freezing, thawing, and sonication as described previously (11). Loading of the hybrid membranes with CF was accomplished by adding CF (50 mM) to the thawed hybrid membranes before sonication. Hybrid membranes were collected by centrifugation for 1 h at  $280,000 \times g_{\text{max}}$  at 4°C, were washed twice with 50 mM K-MES (pH 6.0) to remove nonencapsulated CF, and finally were resuspended in 100  $\mu\text{l}$  of the same buffer.

**Fluorescence measurements.** Fluorescence measurements were determined with a Perkin-Elmer model LS 50 spectrofluorimeter equipped with a thermostat and a continuous stirring device. The efflux of CF from liposomes or hybrid membranes was measured as described previously (15). At an intraliposomal CF concentration of 50 mM, the fluorescence of CF is almost completely self-quenched. Release of CF results in a relief of self-quenching which is recorded as an increase in CF fluorescence with an excitation wavelength of 430 nm (at which the inner-filter effect is negligible

[16]) and an emission wavelength of 520 nm. The excitation and emission slit widths were 2.5 and 5.0 nm, respectively. The experiments were performed at a constant temperature of 25°C. Liposomes (70  $\mu\text{g}$  of lipid per ml) or hybrid membranes (50  $\mu\text{g}$  of protein per ml) were suspended in K-MES buffer (pH 6.0) and treated with pediocin PA-1 or the same volume of solvent (i.e., 20% [vol/vol] ethanol). Complete relief of self-quenching was obtained by adding 0.2% (vol/vol) Triton X-100.

Efflux of high-molecular-mass fluorescein 5-isothiocyanate (FITC)-labeled dextrans (4.4 and 9.4 kDa) was measured as described previously (15). The FITC-labeled dextrans were trapped in fused membranes by adding these components to thawed membranes before sonication. The membranes were collected by centrifugation for 1 h at  $28,000 \times g_{\text{max}}$  at 4°C and were washed twice with 50 mM K-MES buffer (pH 6.0) to remove nonencapsulated dextrans. The membranes containing FITC-labeled dextrans were suspended in 50 mM K-MES buffer (pH 6.0), and the efflux of the dextrans was determined by monitoring the quenching of fluorescein fluorescence by anti-fluorescein rabbit immunoglobulin G (Molecular Probes, Inc., Eugene, Oreg.). Complete release of the encapsulated dextran was obtained by adding 0.2% (vol/vol) Triton X-100. The excitation and emission wavelengths were 490 and 515 nm, respectively. Slit widths of 10.0 nm were used both for excitation and emission.

Tryptophan fluorescence emission spectra were determined with an SLM-AMINCO model 4800C spectrofluorometer (SLM Instruments, Inc., Urbana, Ill.) from 290 to 400 nm by using an excitation wavelength of 280 nm. Slit widths of 4 nm were used, and the spectra were corrected for background fluorescence and averaged ( $n = 10$ ) to reduce the noise value.

**Other analytic procedures.** Protein concentrations in the samples were determined by using the Micro BCA protein assay reagent (Pierce, Rockford, Ill.). CF was purchased from Eastman Kodak Co., Rochester, N.Y., and was purified as described previously (28) by using 50 mM K-MES buffer (pH 6.5) as the eluent.

## RESULTS

**Pediocin PA-1 is bactericidal for sensitive cells.** Pediocin PA-1 inhibits the growth of various bacteria, including the pathogen *Listeria monocytogenes* (34). To determine whether pediocin PA-1 acted on a sensitive test culture of *P. pentosaceus* PPE1.2 wild-type cells in a bacteriostatic, bacteriolytic, or bactericidal manner, the following experiment was conducted. Overnight cultures of wild-type *P. pentosaceus* PPE1.2 (pediocin sensitive) and a pediocin-insensitive derivative carrying the immunity gene for pediocin PA-1 were diluted in fresh MRS broth containing pediocin PA-1 ( $5 \times 10^2$  AU/mg of protein). Pediocin PA-1 affected neither the growth nor the number of CFU of the immune cells (Fig. 1). In contrast, the number of CFU of the sensitive organism decreased dramatically. The optical density of the cell culture remained constant. However, after longer periods of time slight increases in both optical density and number of CFU were observed, possibly as a result of inactivation of the pediocin (3). These results indicate that pediocin PA-1 is bactericidal for sensitive *P. pentosaceus* PPE1.2 cells.

**Pediocin PA-1 dissipates the  $\Delta\psi$  and elicits amino acid efflux from sensitive cells.** Since pediocin PA-1 is a small hydrophobic peptide (20) that resembles other bacteriocins, such as lactococcin A (21), a possible target for its action is the

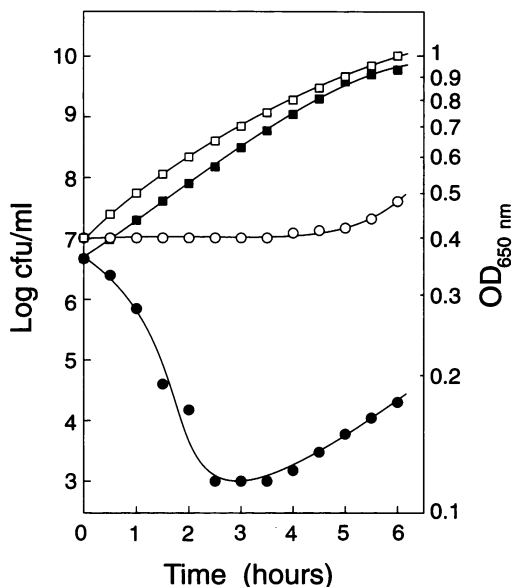


FIG. 1. Effect of pediocin PA-1 on growth and viability of sensitive and immune cells of *P. pentosaceus* PPE1.2. Symbols: ● and ○, pediocin-sensitive *P. pentosaceus* PPE1.2 CFU and optical density at 650 nm ( $OD_{650nm}$ ), respectively; ■ and □, pediocin-insensitive *P. pentosaceus* PPE1.2 CFU and optical density at 650 nm, respectively. The amount of pediocin PA-1 used was 500 AU/mg of protein.

cytoplasmic membrane (46). Therefore, the effects of pediocin PA-1 on the  $\Delta\psi$  of sensitive and immune *Pediococcus* cells were determined.  $\Delta\psi$  (inside negative) was monitored by using an electrode that measured the distribution of the lipophilic cation tetraphenylphosphonium. Pediocin PA-1 (5 AU/mg of protein) caused immediate dissipation of the  $\Delta\psi$  of intact sensitive cells (Fig. 2A), while it had only a minimal effect on the  $\Delta\psi$  of the immune cells (Fig. 2B).

It is very likely that pediocin PA-1 eliminates the  $\Delta\psi$  of sensitive cells by changing the ion permeability of the cytoplasmic membrane. To investigate whether pediocin PA-1 elicited efflux of low-molecular-weight solutes, the effect of pediocin PA-1 on amino acid transport was studied. We studied the uptake of two amino acids which differ in their mechanisms of uptake. AIB, a nonmetabolizable analog of alanine, is accumulated in a  $\Delta p$ -dependent manner (25), whereas L-glutamate is accumulated in a proton motive force ( $\Delta p$ )-independent, most likely ATP-dependent manner (36). Pediocin PA-1 (500 AU/mg of protein) caused the immediate release of AIB accumulated by *P. pentosaceus* PPE1.2 cells sensitive to this bacteriocin (Fig. 3A). In contrast, the ionophore combination valinomycin plus nigericin (1  $\mu$ M each), which completely dissipates the  $\Delta p$  (Fig. 2), caused a slow efflux of AIB. When *P. pentosaceus* PPE1.2 cells were preincubated with pediocin PA-1 or with valinomycin plus nigericin, no uptake of AIB occurred. In contrast, when immune cells were used under the same conditions, no release of AIB was observed when pediocin PA-1 was added (data not shown). Because the inhibition of AIB uptake might result from the dissipation of  $\Delta p$  by pediocin in an indirect way, we examined the effect of pediocin PA-1 on glutamate uptake. *P. pentosaceus* PPE1.2 cells were allowed to accumulate L-glutamate, and subsequently the cells were treated with pediocin PA-1, valinomycin plus nigericin, and all three substances. Accumulated

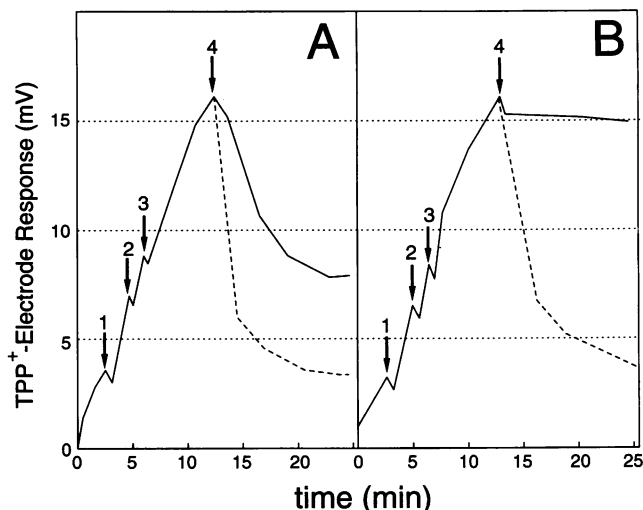


FIG. 2. Effect of pediocin PA-1 on the  $\Delta\psi$  of glucose-energized sensitive (A) and immune (B) cells of *P. pentosaceus* PPE1.2. The effect of the ionophore valinomycin (added at arrow 4) on the uptake of the tetraphenylphosphonium ion ( $TPP^+$ ) is indicated by the dashed line. The arrows indicate the sequential times of addition of cells (arrow 1), glucose (arrow 2), nigericin (arrow 3), and pediocin PA-1 (50 AU/mg of protein) (arrow 4).

glutamate was not released by the ionophores valinomycin and nigericin (Fig. 3B). However, pediocin PA-1 (500 AU/mg of protein) caused the immediate release of the accumulated L-glutamate, both in the presence and in the absence of valinomycin plus nigericin. These data suggest that pediocin PA-1 forms pores in the cytoplasmic membrane that allow the passage of small solutes, such as AIB and glutamate. Moreover, pediocin PA-1 can function in the absence of a  $\Delta p$ , as efflux of glutamate was also induced in the absence of a  $\Delta p$ .

**Pediocin PA-1 induces the release of low-molecular-weight compounds from cytoplasmic membrane vesicles.** To study the pediocin PA-1-induced release of low-molecular-weight compounds in more detail, we used cytoplasmic membrane vesicles derived from sensitive and immune cells. Membrane vesicles derived from sensitive (Fig. 4A) and immune (Fig. 4B) cells of *P. pentosaceus* PPE1.2 rapidly accumulated leucine when a  $\Delta p$  was generated. The  $\Delta p$  was generated by using valinomycin-mediated outwardly directed diffusion gradients of potassium ( $\Delta\psi$ , inside negative) and acetic acid (chemical gradient of protons across the membrane [ $\Delta pH$ ], inside alkaline). When membrane vesicles from pediocin-sensitive cells were preincubated for 5 min in the presence of pediocin PA-1 (1,000 AU/mg of protein), the ability to accumulate leucine was completely lost (Fig. 4A). In contrast, membrane vesicles derived from immune cells accumulated a significant level of leucine (Fig. 4B).

To determine whether pore formation underlies the changes in membrane permeability caused by pediocin, we studied the effect of pediocin PA-1 on leucine counterflow. In these experiments membrane vesicles were loaded with 5 mM leucine and then diluted 50-fold in a medium containing 3.2  $\mu$ M  $^{14}C$ -labeled leucine. Under these conditions, the intravesicular pool of leucine exchanged rapidly with the radiolabeled leucine, which accumulated transiently in response to the outwardly directed leucine concentration gradient (10, 12). The temporary accumulation of radioactive solute does not require  $\Delta p$ . Preincubation of the membrane

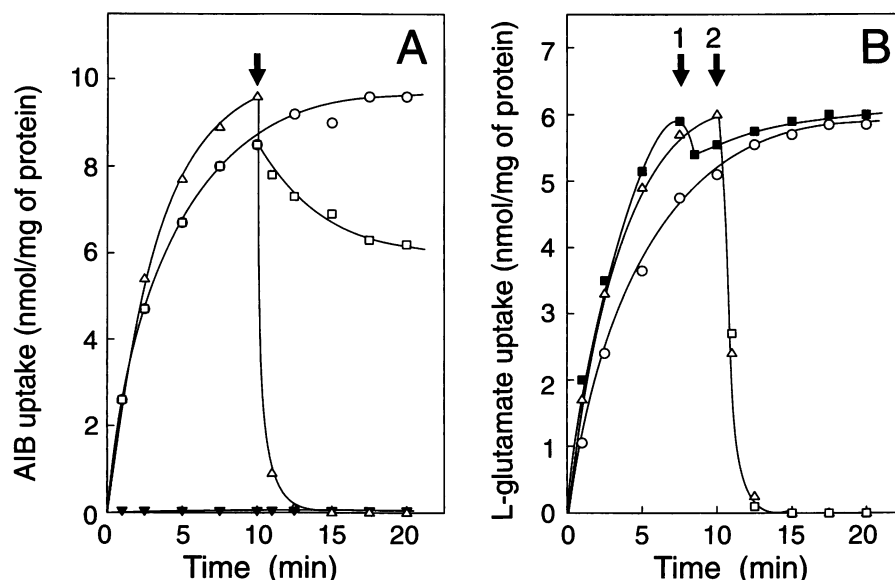


FIG. 3. Effect of pediocin PA-1 on AIB (A) and glutamate (B) uptake in *P. pentosaceus* PPE1.2 cells. (A) Symbols: ○, uptake of AIB by control cells (no additions); □, uptake of AIB in the presence of valinomycin plus nigericin (added at the time indicated by the arrow); Δ, uptake of AIB in the presence of pediocin PA-1 (added at the time indicated by the arrow); ▼, uptake of AIB by cells which were preincubated for 3 min with valinomycin plus nigericin or pediocin PA-1. Cells were energized with glucose. (B) Symbols: ○, uptake of L-glutamate by control cells; ■, uptake of L-glutamate by cells exposed to valinomycin and nigericin (added at the time indicated by arrow 1); Δ, uptake of L-glutamate by cells exposed to pediocin PA-1 (500 AU/mg of protein) (added at the time indicated by arrow 2); □, uptake of L-glutamate by cells exposed to valinomycin plus nigericin and pediocin PA-1 (added at the times indicated by arrows 1 and 2, respectively).

vesicles of the sensitive cells with pediocin PA-1 (1,000 AU/mg of protein) completely destroyed leucine counterflow activity. On the other hand, membrane vesicles of the immune cells exhibited residual leucine counterflow activity when they were incubated with pediocin PA-1 (data not shown). These results suggest that pediocin PA-1 forms pores in isolated cytoplasmic membrane vesicles and that membrane vesicles derived from immune cells are more resistant to pediocin PA-1 than membrane vesicles derived from sensitive cells.

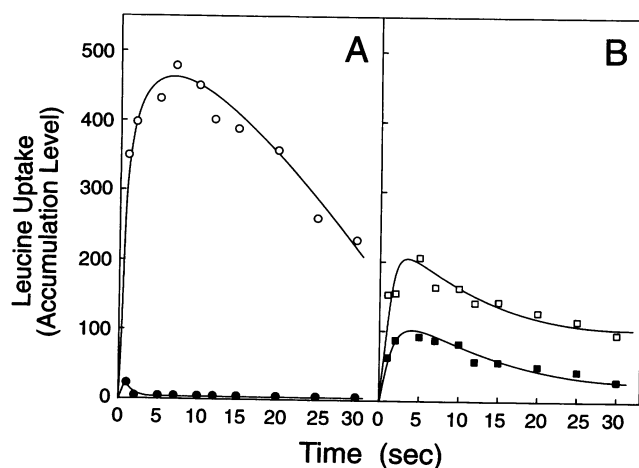


FIG. 4. Effect of pediocin PA-1 on  $\Delta p$ -driven leucine uptake by cytoplasmic membrane vesicles derived from pediocin-sensitive (A) and pediocin-insensitive (B) *P. pentosaceus* PPE1.2. Uptake of leucine by membrane vesicles was determined without (○ and □) or with (● and ■) pediocin PA-1. Membrane vesicles were preincubated with pediocin PA-1 (1,000 AU/mg of protein) for 5 min.

To estimate the size of the pores formed by pediocin, we studied the efflux from membrane vesicles of a number of fluorescent molecules having various molecular weights. The release of membrane vesicle-entrapped CF is a convenient and sensitive technique which allowed us to perform an assay of pore formation in real time. CF present at a high concentration in the membrane vesicle lumina exhibited a low level of fluorescence because of a high degree of self-quenching. Release of CF was detected as relief of self-quenching, as shown by an increase in CF fluorescence. To load the membrane vesicles with CF, the vesicles were fused with liposomes composed of phospholipids extracted from *P. pentosaceus* cells. Liposomes loaded with CF were completely resistant to pediocin, as no increase in CF fluorescence was observed when pediocin (5,000 AU/mg of protein) was added (Fig. 5). However, rapid release of CF when pediocin PA-1 was added was observed in the hybrid membranes prepared with membrane vesicles of both sensitive and immune cells (Fig. 5).

A similar approach was used to analyze the pediocin PA-1-induced efflux of 4.4-kDa FITC-labeled dextran (data not shown) and 9.4-kDa FITC-labeled dextran (Fig. 6). Release of FITC-labeled dextran from the hybrid membranes prepared from sensitive membrane vesicles was monitored with anti-fluorescein antibodies which, when present on the outside, quenched the fluorescence of released FITC-labeled dextrans. When pediocin was present at sufficiently high concentrations (2,000 AU/mg of protein), a rapid release of the FITC-labeled dextrans was observed (Fig. 6). At higher pediocin concentrations (5,000 AU/mg of protein), the efflux of FITC-labeled dextrans was more pronounced. These results suggest that pediocin PA-1 can form large pores that allow membrane passage of high-molecular-weight compounds. Moreover, these results suggest that more pediocin is required to release compounds

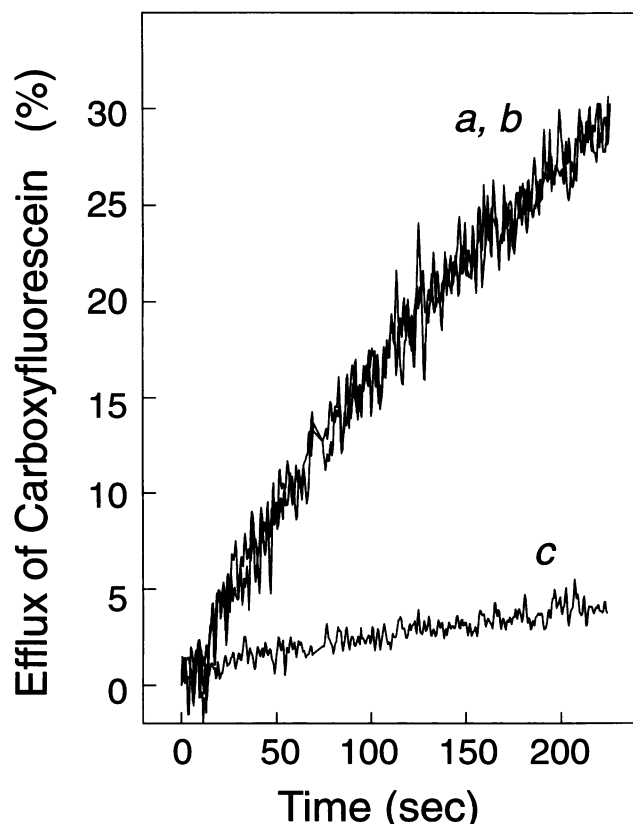


FIG. 5. Pediocin PA-1-induced efflux of CF from *P. pentosaceus* membrane vesicles fused with liposomes. Liposomes prepared from *P. pentosaceus* PPE1.2 phospholipids (line c) were fused with membrane vesicles derived from pediocin-sensitive (line a) and pediocin-insensitive (line b) cells in the presence of CF. Pediocin PA-1 (5,000 AU/mg of protein) was added at time zero.

having higher molecular weights (Fig. 1 through 6). We concluded that the cytoplasmic membrane is the target of pediocin activity. However, sensitivity to pediocin requires a factor(s) which is not present in liposomes prepared from phospholipids derived from sensitive *P. pentosaceus* cells.

**DTT inhibits pediocin PA-1 activity.** The mature region of pediocin PA-1 contains four cysteine residues which form two disulfide bonds (20). The possible function of these disulfide bonds in pediocin activity was studied by reducing the peptide in the presence of dithiothreitol (DTT). DTT-treated pediocin PA-1 had completely lost the ability to kill sensitive cells when it was analyzed in a spot test (data not shown).

Pediocin PA-1 contains two tryptophan residues at positions 19 and 33 and two tyrosine residues at positions 2 and 3. This allowed us to observe qualitative changes in the tertiary structure of the molecule by examining tryptophan fluorescence. When pediocin PA-1 was unfolded in 6 M guanidine-HCl (Fig. 7), only minor changes in the tryptophan fluorescence spectrum were observed compared with pediocin diluted in buffer. These data suggest that the tryptophan (and tyrosine) residues of pediocin in buffer are exposed to solvent, indicating that the molecule has an unfolded or loose conformation. When pediocin PA-1 was incubated in the presence of *Escherichia coli* liposomes, the tryptophan fluorescence emission spectrum was broadened

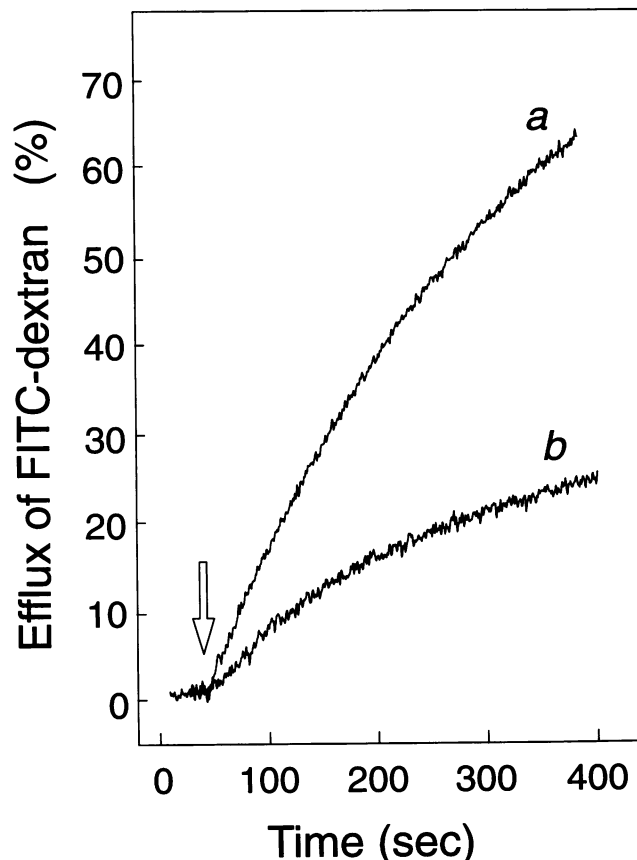


FIG. 6. Pediocin PA-1-induced efflux of FITC-labeled dextran from *P. pentosaceus* membrane vesicles fused with liposomes. Membrane vesicles from pediocin-sensitive cells were fused with liposomes prepared from *P. pentosaceus* PPE1.2 phospholipids in the presence of FITC-labeled dextran with an average molecular mass of 9.4 kDa. The time of addition of pediocin PA-1 (line a, 5,000 AU/mg of protein; line b, 2,000 AU/mg of protein) is indicated by the arrow. The accessibility of the FITC moiety to externally added anti-fluorescein antibodies was monitored by measuring the decrease in fluorescein fluorescence.

with a blue shift of the emission maximum. These data suggest that the tryptophan residues were transferred from a polar environment to a nonpolar environment, possibly as a result of membrane insertion or a lipid-induced conformational change of the molecule. Similar changes in the fluorescence spectrum were obtained when pediocin PA-1 was incubated in the presence of DTT, although the maximum fluorescence was decreased because of inner-filter effects caused by near-UV absorbance by DTT (data not shown). Quenching of the tryptophan fluorescence by water-soluble acrylamide was more efficient with the soluble pediocin (Fig. 7, inset) than with pediocin incubated in the presence of lipids. To determine the depth of pediocin penetration into the lipid bilayer, we prepared liposomes containing fatty acyl chain doxyl-derivatized phosphatidylcholine analogs with the doxyl moiety at various positions in the stearyl fatty acyl chain. The doxyl moiety is an efficient quencher of tryptophan fluorescence provided that the tryptophan residues of pediocin are close to the doxyl group. The fluorescence levels were 0.83, 0.90, and 0.92 with the doxyl moiety at positions 5, 10, and 16, respectively in the stearyl fatty acyl chain (data not shown). These results suggest that the

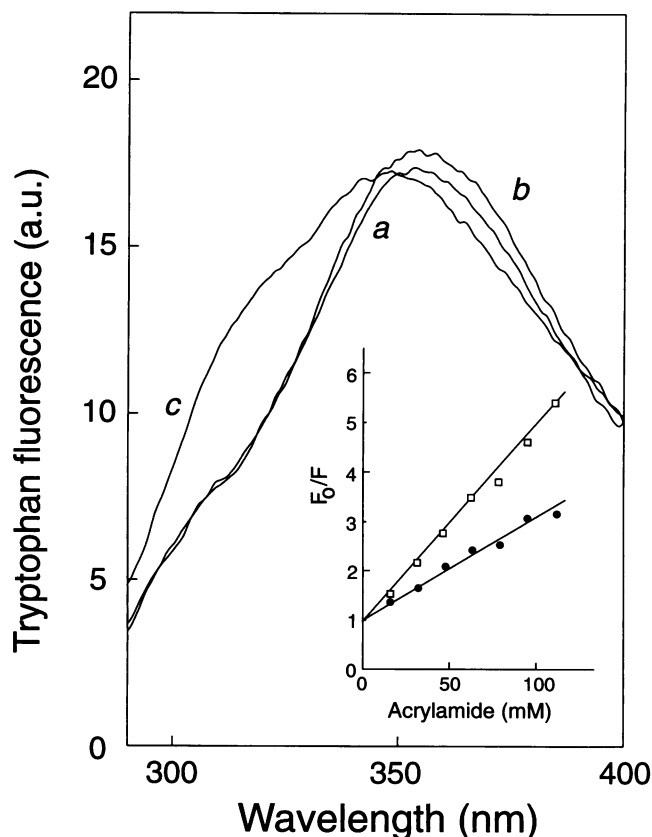


FIG. 7. Tryptophan fluorescence of pediocin PA-1. Pediocin PA-1 was incubated in 50 mM K-MES buffer (pH 6.0) in the absence (line *a*) or presence (line *c*) of liposomes composed of *E. coli* phospholipids (40  $\mu$ g) in a final volume of 110  $\mu$ l. Line *b* is the tryptophan fluorescence spectrum of pediocin unfolded in 6 M guanidine-HCl-50 mM K-MES buffer (pH 6.0). (Inset) Stern-Volmer plot of the quenching of tryptophan fluorescence of pediocin PA-1 by acrylamide in the absence (●) and presence (□) of liposomes composed of *E. coli* phospholipids. Fluorescence emission was measured at 320 nm.

tryptophan residues of pediocin PA-1 are localized on the surface when pediocin PA-1 is associated with liposomes. The peptide may assume a more ordered structure upon interaction with the membrane surface irrespective of the reduction state of its disulfide bonds.

## DISCUSSION

Pediocin PA-1 belongs to a group of bacteriocins produced by *Pediococcus* spp. (7, 24, 29, 39). In this study we examined the mode of action of pediocin PA-1. Our data suggest that pediocin PA-1 kills sensitive cells by effecting the release of metabolites by the formation of pores in the target membranes.

Recently, the amino acid sequence of pediocin AcH was described on the basis of both an amino acid sequence determination and the sequence derived from the nucleotide sequence of the gene (33). Pediocins AcH and PA-1 have identical sequences. The protein is highly hydrophobic and would be expected to interact with membranes (41). In addition, several other bacteriocins exhibit high levels of amino acid sequence homology with pediocin PA-1 (4, 30, 32). The results of our preliminary studies on the effect of the

bacteriocins sakacin A (curvacin A) and sakacin P (4, 45) on glutamate release from *P. pentosaceus* PPE1.2 indicate that these bacteriocins are also able to cause the release of glutamate under conditions in which the  $\Delta p$  was dissipated by the ionophores valinomycin and nigericin (6). These bacteriocins may function like pediocin PA-1. Bhunia et al. (3) showed that pediocin AcH binds with high efficiency to the surfaces of cells in a nonspecific manner. On the basis of the data presented by Bhunia et al. (3) and our results, we propose the following working model for the mechanism of action of pediocin PA-1 (AcH). Pediocin molecules first adhere nonspecifically to the surfaces of target cells, and this is followed by binding to a receptor-like component of the cell membrane. The pediocins may then insert into the membrane and aggregate into oligomeric structures. These structures then form hydrophilic pores which allow the release of ions and small molecules from the target cells; ultimately, this leads to cell death, with or without lysis. Our *in vitro* studies revealed that at high concentrations pediocin functions in the absence of a proton motive force across the membrane. In contrast to the lantibiotic nisin (15), imposition of a  $\Delta p$  did not affect the efficiency with which pediocin caused release of fluorescent molecules from hybrid membranes (6). We cannot exclude the possibility that *in vivo*  $\Delta p$  contributes to the efficiency with which pediocin at a low concentration acts on the target cell membranes.

Pediocin PA-1 acts in a bactericidal manner on sensitive *P. pentosaceus* PPE1.2 cells. It may also exert a bacteriolytic effect, depending on the species of the sensitive cells (3). The activities of pediocin PA-1 in the different systems tested strongly depend on the concentration used. Similar results have been reported for lactococcin A (46), lactococcin B (47), and pediocin JD (7). The latter bacteriocin may be identical to pediocin PA-1 (AcH). Our data suggest that the concentration of pediocin determines the size exclusion limit of the pores. Only at higher concentrations was effective release of the high-molecular-weight dextrans observed, while lower concentrations of pediocin were sufficient to dissipate the  $\Delta p$ . The lantibiotic nisin, however, does not cause the release of dextrans from liposomes (15).

Reduction of the disulfide bonds in pediocin PA-1 resulted in a complete loss of bacteriocin activity. It has been reported that the cysteine residues present in sakacin A are in a reduced state (4), while the cysteine residues in pediocin PA-1 are in an oxidized state, forming disulfide bonds (20). Two cysteine residues of pediocin PA-1, sakacin A (curvacin A), and sakacin P are in the same positions (i.e., at position 9 or 10 and at position 14 or 15) in the primary structures of the aligned sequences. The additional cysteine residues of pediocin PA-1 at positions 24 and 44 are not present in sakacin A and sakacin P. Since DTT does not inhibit the antimicrobial activities of sakacin A and sakacin P (6), it seems likely that the disulfide bond between the cysteine residues at positions 24 and 44 is essential for pediocin PA-1 activity.

Pediocin PA-1 is ineffective against liposomes composed of *P. pentosaceus* PPE1.2 phospholipids, but it does affect hybrid membrane vesicles of this organism fused with liposomes composed of both *P. pentosaceus* and *E. coli* phospholipids (6). *E. coli* cells are not sensitive to pediocin PA-1. These data suggest that there is a requirement for a protein in the target membrane, as suggested for lactococcin A (46), a bacteriocin produced by certain *Lactococcus lactis* strains, rather than for a lipid component. *Pediococcus* cells which synthesize the pediocin immunity protein are highly resistant to pediocin PA-1 treatment. This property is partially pre-



served in membrane vesicles derived from immune cells. A similar phenomenon has been observed with lactococcin A (46). On the other hand, the immunity phenotype appears to be lost when these membrane vesicles are fused with liposomes composed of *Pediococcus* phospholipids. The reason for this observation is not known. Loss of the resistant phenotype could be a result of inactivation or loss of the immunity protein in the fused membranes. Proteinase K treatment of membrane vesicles derived from both immune and sensitive cells resulted in increased resistance of the membrane vesicles of sensitive cells and decreased resistance of membrane vesicles of immune cells (6). This implies that there is a complicated mechanism of immunity in which the immunity protein is not the only protein that participates.

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#### REFERENCES

- Berry, E. D., M. B. Liewen, R. W. Mandigo, and R. W. Hutkins. 1990. Inhibition of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus* during the manufacture of fermented semidry sausages. *J. Food Prot.* **53**:194-197.
- Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *J. Appl. Bacteriol.* **65**:261-268.
- Bhunia, A. K., M. C. Johnson, B. Ray, and N. Kalchayanand. 1991. Mode of action of pediocin ACH from *Pediococcus acidilactici* H in sensitive bacterial strains. *J. Appl. Bacteriol.* **70**:25-33.
- Blom, H. 1993. Personal communication.
- Bruno, M. E. C., A. Kaiser, and T. J. Montville. 1992. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. *Appl. Environ. Microbiol.* **58**:2255-2259.
- Chikindas, M. L. 1992. Unpublished data.
- Christensen, D. P., and R. W. Hutkins. 1992. Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. *Appl. Environ. Microbiol.* **58**:3312-3315.
- Daeschel, M. A., and T. R. Klaenhammer. 1985. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. *Appl. Environ. Microbiol.* **50**:1538-1541.
- Driessen, A. J. M. 1987. Amino acid transport in lactic streptococci. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
- Driessen, A. J. M., J. Kodde, S. de Jong, and W. N. Konings. 1987. Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subjected to regulation by internal pH. *J. Bacteriol.* **169**:2748-2754.
- Driessen, A. J. M., and W. N. Konings. 1993. Insertion of lipids and proteins into bacterial membranes by fusion with liposomes. *Methods Enzymol.* **221**:394-408.
- Driessen, A. J. M., T. Zhen, G. In't Veld, J. Op den Kamp, and W. N. Konings. 1988. The lipid requirement of the branched chain amino acid carrier of *Streptococcus cremoris*. *Biochemistry* **27**:865-872.
- Fitzsimons, A., F. Duffner, D. Curtin, G. Brophy, P. O'Kiely, and M. O'Connell. 1992. Assessment of *Pediococcus acidilactici* as a potential silage inoculant. *Appl. Environ. Microbiol.* **58**:3047-3052.
- Gao, F. H., T. Abee, and W. N. Konings. 1991. The mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome *c* oxidase proteoliposomes. *Appl. Environ. Microbiol.* **57**:2164-2170.
- García-Garcera, M. J., M. G. L. Elferink, A. J. M. Driessen, and W. N. Konings. 1993. *In vitro* pore forming activity of the antibiotic nisin: role of protonmotive-force and lipid composition. *Eur. J. Biochem.* **212**:417-422.
- Goessens, W. H. F., A. J. M. Driessen, J. Wilschut, and J. van Duin. 1988. A peptide corresponding to the C-terminal 25 residues of phage MS2 coded lysis protein dissipates the membrane potential in *Escherichia coli* vesicles by generating hydrophilic pores. *EMBO J.* **7**:867-873.
- Gonzalez, C. F., and B. S. Kunka. 1986. Evidence for plasmid linkage of raffinose utilization and associated  $\alpha$ -galactosidase and sucrose hydrolase activity in *Pediococcus pentosaceus*. *Appl. Environ. Microbiol.* **51**:105-109.
- Gonzalez, C. F., and B. S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. *Appl. Environ. Microbiol.* **53**:2534-2538.
- Graham, D. C., and L. L. McKay. 1985. Plasmid DNA in strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus*. *Appl. Environ. Microbiol.* **50**:532-534.
- Henderson, J. T., A. L. Chopko, and P. D. van Wassenaar. 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC1.0. *Arch. Biochem. Biophys.* **295**:5-12.
- Holo, H., O. Nilssen, and I. F. Nes. 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. *J. Bacteriol.* **173**:3879-3887.
- Hoover, D. G., P. M. Walsh, K. M. Kolaetis, and M. M. Daly. 1988. A bacteriocin produced by *Pediococcus* species associated with a 5.5 megadalton plasmid. *J. Food Prot.* **51**:29-31.
- Kagawa, Y., A. Kandrach, and E. Racker. 1973. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **248**:676-684.
- Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* **70**:337-349.
- Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics and solute transport in lactococci. *Crit. Rev. Microbiol.* **16**:419-476.
- Konisky, J. 1982. Colicin and other bacteriocins with established modes of action. *Annu. Rev. Microbiol.* **36**:125-144.
- Kordel, M., R. Benz, and H.-G. Sahl. 1986. Susceptibility of bacterial, eucaryotic and artificial membranes to the disruptive action of the cationic peptides Pep5 and nisin. *FEMS Microbiol. Lett.* **34**:139-144.
- Lelkes, P. I. 1984. A simple one-step purification method for 5,6-carboxyfluorescein, p. 225. In G. Gregoriachis (ed.), *Liposome technology*. CRC Press, Boca Raton, Fla.
- Lewus, C. B., A. Kaiser, and T. J. Montville. 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environ. Microbiol.* **57**:1683-1688.
- Lozano, J. C. N., J. N. Meyer, K. Sletten, C. Pelaz, and I. F. Nes. 1992. Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici*. *J. Gen. Microbiol.* **138**:1985-1996.
- Luchansky, J. B., K. A. Glass, K. D. Harsono, A. J. Degnan, N. G. Faith, B. Cauvin, G. Baccus-Taylor, K. Arihara, B. Bater, A. J. Maurer, and R. G. Cassens. 1992. Genomic analysis of *Pediococcus* starter cultures used to control *Listeria monocytogenes* in turkey summer sausage. *Appl. Environ. Microbiol.* **58**:3053-3059.
- Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledebuer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenberg. 1992. Cloning, expression and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. *Appl. Environ. Microbiol.* **58**:2360-2367.
- Motlagh, A. M., A. K. Bhunia, F. Szostek, T. R. Hansen, M. C. Johnson, and B. Ray. 1992. Nucleotide and amino acid sequence of *pap*-gene (pediocin ACH production) in *Pediococcus acidilactici* H. *Lett. Appl. Microbiol.* **15**:45-48.
- Nielsen, J. W., J. S. Dickson, and J. D. Crouse. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Appl. Environ. Microbiol.* **56**:2142-2145.



35. Pederson, C. S. 1949. The genus *Pediococcus*. Bacteriol. Rev. 13:225–232.
36. Poolman, B., E. J. Smid, and W. N. Konings. 1987. Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*. J. Bacteriol. 169:1460–1468.
37. Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenberg. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. 54:2349–2353.
38. Ray, B., and M. Daeschel. 1992. Food biopreservatives of microbial origin. CRC Press, Inc., Boca Raton, Fla.
39. Ray, S. K., M. C. Johnson, and B. Ray. 1989. Bacteriocin plasmids of *Pediococcus acidilactici*. J. Ind. Microbiol. 4:163–171.
40. Ruhr, E., and H.-G. Sahl. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. 27:841–845.
41. Schiffer, M., C.-H. Chang, and F. G. Stevens. 1992. The function of tryptophan residues in membrane proteins. Protein Eng. 5:213–214.
42. Schlech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome. 1983. Epidemic listeriosis—evidence for transmission by food. N. Engl. J. Med. 308:203–206.
43. Shinbo, T., N. Kama, K. Kurihara, and Y. Kobataka. 1978. PVC-based electrode sensitive to DDA<sup>+</sup> as a device to monitor the membrane potential in biological systems. Arch. Biochem. Biophys. 187:414–422.
44. Smith, J. L., and S. A. Palumbo. 1983. Use of starter cultures in meats. J. Food Prot. 46:997–1006.
45. Tichaczek, P. S., J. Nissen-Meyer, I. F. Nes, R. F. Vogel, and W. P. Hammes. 1992. Characterization of the bacteriocin curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *L. sake* LTH673. Syst. Appl. Microbiol. 15:460–468.
46. van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee. 1991. The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J. Bacteriol. 173:7934–7941.
47. Venema, K., T. Abee, A. J. Haandrikman, K. J. Leenhouts, J. Kok, W. N. Konings, and G. Venema. 1993. Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. 59:1041–1048.
48. WHO Working Group. 1988. Foodborne listeriosis. Bull. W.H.O. 66:421–428.
49. Yousef, A. E., J. B. Luchansky, A. J. Degnan, and M. P. Doyle. 1991. Behavior of *Listeria monocytogenes* in wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin AcH during storage at 4 or 25°C. Appl. Environ. Microbiol. 57:1461–1467.
50. Zadjel, J. L., P. Ceglowski, and W. T. Dobrzanski. 1985. Mechanism of action of lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris* 202. Appl. Environ. Microbiol. 49:969–974.